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(54) Title: SYNTHETIC MULTIMERIC PEPTIDE WITH DELTA HEPATITIS VIRUS ANTIGENIC ACTIVITY

(57) Abstract

Synthetic peptides exhibit delta hepatitis virus antigenic activity and self-assembly that results in the formation of large multimers and display of a conformational epitope. The peptides may be used to confer protective immunity in subjects or to raise antibodies for diagnostic purposes. Diagnostic assays and kits for diagnostic assays utilize these peptides.

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SYNTHETIC MULTIMERIC PEPTIDE WITH DELTA HEPATITIS VIRUS ANTIGENIC ACTIVITY

This invention was made with Government support under grants GM42031 and HL37974 from the U.S. Public Health Service. The Government has certain rights to this invention.

Field of the Invention

This application concerns synthetic peptides that exhibit delta hepatitis virus antigenic activity and self-assembly activity which results in the formation of large multimers, and methods of use thereof.

Background of the Invention

Hepatitis delta virus (HDV) is unique among animal viruses. This subviral satellite depends upon a co-infecting hepadnavirus for provision of its envelope and often causes severe and even fatal liver disease in humans. *See* Rizzetto, M. (1983) *Hepatology* 3:729-737; Hoofnagle, J. H. (1989) *J. Am. Med. Assoc.* **261**: 1321-1325. The hepatitis delta antigen (HDAg) is the only protein expressed from its 1.7-kb circular RNA genome. *See* Weiner, A. J., *et al.* (1988) *J. Virol.* **62**: 594-599; *see also* European Patent Application of Choo *et al.*, EP 251575 (Chiron Corp.) (nucleotide sequence of the hepatitis delta virus genome). This nuclear phosphoprotein exists in two forms that have contrasting functions in virus replication. Weiner et al., *supra*. The 195-residue small form is a trans-acting promoter of replication while the 214-residue large form is a down-regulator of replication and may promote HDV particle assembly. *See* Kuo, M. Y.-P., *et al.*, (1989) *J. Virol.* **63**:1945-1950. Both forms specifically bind HDV RNA, translocate to the nucleus, and associate into homodimeric, heterodimeric, and larger multimeric structures. *See* Chang, M.-F., *et al.* (1988) *J. Virol.* **62**:2403-2410; Lin, J.-H., *et al.* (1988) *J. Virol.* **64**:4051-4058. Self-association of HDAg into multimers is required for full biological activity, but its

mechanism is obscure. *See* Xia, Y.-P. & Lai, M. M. C., (1992) *J. Virol.* **66**:6641-6648.

The N-terminal half of HDAg is involved in the formation of dimers and multimers of HDAg. In vitro translation products representing the N-terminal third of HDAg formed dimers that were detected by chemical crosslinking. *Id.* Similarly, chymotryptic fragments containing the N-terminal 76-81 residues of HDAg recovered from infected liver formed dimers and multimers. *See* Wang, J.-G. & Lemon, S. M. (1993) *J. Virol.* **67**:446-454. Deletions or point mutation of Leu37, Leu44, and Ile41 variously to glycine, valine or proline impaired the replication-related functions of HDAg, presumably by disrupting dimer formation. The dimerization of HDAg may involve formation of an α-helical coiled-coil, which is characterized by a seven-residue repeating pattern (heptad) in which the first and fourth residues are hydrophobic. *See* Landschulz, W. H., et al., (1988) *Science* **240**:1759-1764. A well conserved heptad pattern of leucine and isoleucine residues is located within the N-terminal third of HDAg between Leu27 and Ile58. However, the computer algorithm of Lupas *et al.* (*Science* **252**, 1162-1164 (1991)) predicts that the residues from Leu17 to Pro49 should form a coiled-coil. This segment of HDAg contains two conserved residues (Gly23 and Pro49) that may distort an alpha helix and thus divide the region spanning residues 12-60 into three segments: A(Gly^{12}-Arg^{24}), B(Lys^{25}-Pro^{49}), and C(Trp^{50}-Lys^{60}).

Antigen currently employed in commercial immunoassays for antibodies to hepatitis delta antigen is prepared from infected woodchuck liver. A need exists for a more stable, homogeneous and economical source of antigen for use in such assays.

Summary of the Invention

A peptide (δ12-60(Y)) (SEQ ID NO: 1), has been designed and synthesized which possesses significant delta virus antigenic activity. The peptide was designed

to mimic a coiled-coil dimerization domain within the amino terminal third of the hepatitis delta virus protein. Circular dichroism spectroscopy has demonstrated that this peptide (a 50mer) has a strong concentration-dependent tendency to form α-helical coiled-coil complexes, with a Tm (temperature at the midpoint of thermal denaturation) in excess of 80°C. Solid-phase immunoassays in several formats suggest that this peptide demonstrates exceptionally strong and broadly reactive antigenic activity, and expresses conformational epitopes of hepatitis delta antigen. Additional evidence suggests that it self-assembles into a multimeric structure composed of four or more peptide chains. Other assays indicate that this peptide is able to form heterodimers with native delta virus proteins and thereby disrupt normal multimerization of hepatitis delta virus antigen.

Disclosed are synthetic peptides having delta hepatitis virus antigenic activity, including the synthetic peptides δ12-60(Y) (SEQ ID NO:1), δ12-60(Y)/S22C (SEQ ID NO:4), δ12-60(Y)/CAR (SEQ ID NO:5), δ12-60(Y)/Fr (SEQ ID NO:6), δ12-60(Y)/It1 (SEQ ID NO:7), δ12-60(Y)/It2 (SEQ ID NO:8), δ12-60(Y)/Ja1 (SEQ ID NO:9), δ12-60(Y)/Ja2 (SEQ ID NO:10), δ12-60(Y)/Le (SEQ ID NO:11), δ12-60(Y)/Na (SEQ ID NO:12), δ12-60(Y)/Pe (SEQ ID NO:13), δ12-60(Y)/Ta (SEQ ID NO:14), and δ12-60(Y)-Cons (SEQ ID NO:15); and peptides at least 40 amino acids in length that form a heteromer or a homomer with peptide δ12-60(Y).

Also disclosed is a method for detecting the presence of antibodies that bind to hepatitis delta virus antigen. The method comprises contacting a biological sample taken from a subject with an antigen, where the antigen is a synthetic peptide having delta hepatitis virus antigenic activity, under conditions permitting the formation of an antibody-antigen complex. The amount of antibody-antigen complex in the sample is a measure of

the amount of antibody in the sample. The synthetic peptid may be any of peptides δ12-60(Y) (SEQ ID NO:1), δ12-60(Y)/S22C (SEQ ID NO:4), δ12-60(Y)/CAR (SEQ ID NO:5), δ12-60(Y)/Fr (SEQ ID NO:6), δ12-60(Y)/It1 (SEQ ID NO:7), δ12-60(Y)/It2 (SEQ ID NO:8), δ12-60(Y)/Ja1 (SEQ ID NO:9), δ12-60(Y)/Ja2 (SEQ ID NO:10), δ12-60(Y)/Le (SEQ ID NO:11), δ12-60(Y)/Na (SEQ ID NO:12), δ12-60(Y)/Pe (SEQ ID NO:13), δ12-60(Y)/Ta (SEQ ID NO:14), and δ12-60(Y)-Cons (SEQ ID NO:15).

Kits useful for detecting hepatitis delta virus antibodies are also disclosed. The kits comprise an antigen as described above, which antigens may be immobilized on said solid support.

Also disclosed is a method of producing antibodies to delta hepatitis virus in a mammalian subject, comprising administering to the subject an immunogenic amount of a peptide as described above.

Also disclosed is a method of immunizing a mammalian subject against delta hepatitis virus infection. The method comprises administering to the subject an immunogenic amount of a peptide as described above.

The foregoing and other objects and aspects of the present invention are explained in detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

FIG. 1A is a graph of ELISA results of plasma specimens from 23 HbsAG-positive patients with anti-HD antibodies detectable by a commercially available assay, wherein the ELISA utilized the peptide δ12-60(Y) (SEQ ID NO:1).

FIG. 1B is a graph of ELISA results of plasma specimens from 31 HbsAG-negative patients without anti-HD antibodies, wherein the ELISA utilized the peptide δ12-60(Y) (SEQ ID NO:1).

FIG. 1C is a graph of ELISA results of plasma specimens from 35 HbsAG-positive patients without anti-HD

antibodies detectable by a commercially available assay, wherein the ELISA utilized the peptide δ12-60(Y) (SEQ ID NO:1).

FIG. 2A is a graph of ELISA results using bound murine anti-HDAg monoclonal antibodies 4A5 and 6H8, tested for reactivity against synthetic peptides having sequences based on the hepatitis delta virus antigen.

FIG. 2B is a graph of ELISA results using bound murine anti-HDAg monoclonal antibodies 3G3 and 8B3, tested for reactivity against synthetic peptides having sequences based on the hepatitis delta virus antigen.

Detailed Description of the Invention

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. Amino acids are represented herein by three letter code, in accordance with 37 CFR §1.822 and established usage. *See, e.g.*, PatentIn User Manual, 99-102 (Nov. 1990)(U.S. Patent and Trademark Office, Office of the Assistant Commissioner for Patents, Washington, D.C. 20231); U.S. Patent No. 4,871,670 to Hudson et al. at Col. 3 lines 20-43 (applicants specifically intend that the disclosure of this and all other patent references cited herein be incorporated herein by reference).

1. Peptides

This invention discloses a large synthetic peptide of 50 residues (δ12-60(Y)) (SEQ ID NO:1) representing a self-assembly domain of the hepatitis delta virus antigen (HDAg which is 195-214 residues in length). This synthetic peptide has self-assembly activity which results in the formation of large multimers (4-6 copies of the peptide) which have very strong antigen activity not present in smaller peptides made from this same region. The mechanism of assembly is probably unique, and cannot be predicted by any available

program. Self-assembly allows it to mimic a conformationally determined epitope - something peptides rarely do.

Peptides of the present invention include analogs of the peptide of SEQ ID NO:1. As used herein, analogs are those compounds which, while not having amino acid sequences identical to those of the peptides described above, have a similar three-dimensional structure. In protein molecules which interact with a receptor or complementary determining region of an immunoglobulin molecule, the interaction between the protein and the receptor must take place at the surface-accessible sites in a stable three-dimensional molecule. By arranging the critical binding site residues in an appropriate conformation, peptides which mimic the essential surface features of the peptides of the present invention are designed and synthesized in accordance with known techniques. Methods for determining peptide three-dimensional structure and analogs thereto are known, and are sometimes referred to as "rational drug design techniques". *See, e.g.*, U.S. Patent No. 4,833,092 to Geysen; U.S. Patent No. 4,859,765 to Nestor; U.S. Patent No. 4,853,871 to Pantoliano; U.S. Patent No. 4,863,857 to Blalock (applicants specifically intend that the disclosures of all U.S. Patent references cited herein be incorporated by reference herein in their entirety). *See also* Waldrop, *Science*, **247**, 28029 (1990); Rossmann, *Nature*, **333**, 392-393 (1988); Weis et al., *Nature*, **333**, 426-431 (1988). Techniques for constructing and screening libraries of peptide sequences to identify peptides that specifically bind to a given protein are known. Scott and Smith, *Science*, **249**, 386-390 (1990); Devlin et al., *Science*, **249**, 404-406 (1990). Further, those skilled in the art will appreciate that minor deletions or substitutions may be made to the amino acid sequences of peptides of the present invention without unduly adversely affecting the activity thereof. Thus,

peptides containing such deletions or substitutions are a further aspect of the present invention. In addition, the C-terminal Tyr was added to facilitate labelling, and can be deleted from the sequence without impairing multimer assembly or antigenicity.

In peptides containing substitutions or replacements of amino acids, one or more amino acids of a peptide sequence may be replaced by one or more other amino acids which do not affect the antigenicity of that sequence. Such changes can be guided by known similarities between amino acids in physical features such as charge density, hydrophobicity/hydrophilicity, size and configuration, so that amino acids are substituted with other amino acids having essentially the same functional properties. For example:

Ala may be replaced with Val, Gly or Ser, preferably Ser;

Val may be replaced with Ala, Cys, Leu, Met, or Ile, preferably Ala or Ile;

Ile may be replaced with Cys, Ala, Val or Leu, preferably Val or Leu;

Leu may be replaced with Cys, Ala, Val or Ile, preferably Val or Ile;

Gly may be replaced with Ser, Cys or Ala, preferably Ala;

Pro may be replaced with Gly or Ser;

Cys may be replaced with Gly, Ala or Ser, preferably Ser;

Met may be replaced with Leu or Ile, preferably Leu;

His may be replaced with Lys, Arg, Phe or Gln, preferably Gln;

Phe may be replaced with His, Tyr, or Trp, preferably Tyr;

Tyr may be replaced with His, Phe or Trp, preferably Phe or Trp;

Trp may be replaced with Phe or Tyr, preferably Tyr;

Asn may be replaced with Asp, Glu, Gln or Ser, preferably Gln;

Gln may be replaced with His, Lys, Glu, Asn, or Ser, preferably Asn or Ser;

Ser may be replaced with Gln, Thr, Cys, or Ala;

Thr may be replaced with Gln or Ser, preferably Ser;

Lys may be replaced with Gln, Arg, Asp, or Glu, preferably Arg;

Arg may be replaced with Lys, Asp or Glu, preferably Lys;

Asp may be replaced with Lys, Arg, or Glu, preferably Glu; and

Glu may be replaced with Arg, Lys or Asp, preferably Asp.

The effects of such changes on antigenicity can be determined by routine screening with antibodies which are known to bind to the antigen.

Peptides of the present invention bind to one another to form multimers (*e.g.*, dimers, tetramers and/or hexamers). Several techniques can be used to determine the multimerization state of a given peptide (homomer) or peptide mixture (heteromer). The most straightforward methods involve determining the apparent molecular weight of the multimer complex and from this determining the number of associated monomer components (this can be accomplished by dividing this apparent molecular weight by the molecular weight of the monomer). Analytical ultracentrifugation is a particularly suitable technique for this purpose. The specifics of this method are known to those skilled in the art. *See, e.g.*, P. Graceffa et al., *J. Biol. Chem.* **263**, 14196-14202 (1988), and can be summarized as follows. The material of interest is placed in a sample cell and spun very rapidly in a model

E ultracentrifuge equipped with the appropriate detection devices. Information collected during the experiment combined with the amino acid composition of the peptide allows for the determination of the apparent MW of the multimer complex. Fast Protein Liquid Chromatography (FPLC) can also be used for this purpose. This technique is different from the above in that, as a type of chromatography, it ultimately requires reference to some primary standard (determined by analytical ultracentrifugation). Pharmacia Biosystems supplies the SUPERDEX 75™ column, which allows for the separation of various multimeric forms of self-associating peptides on the basis of differences in total mass. These determinations are carried out under non-denaturing (native) conditions and, when referenced to the appropriate standards, can be used to identify peptide and protein oligomerization states.

As will also be apparent to those skilled in the art, the test for heterodimerization may be carried out using either of the above two methods or through the use of CD combined with one or the other of these methods. This latter technique, in brief, involves adding known amounts of peptide to a solution containing a known amount of either the same peptide (for homodimerization) or a different peptide (for heterodimerization) and following the CD signal as a function of this addition. An increase in the magnitude of the signal as peptide is added indicates that the added material is participating in multimer formation. Homo vs heterodimerization is determined by carrying out this same experiment using FPLC or ultracentrifugation, which would determine if the resulting system is either a single heteromer or multiple homomers. A second, and particularly preferred, approach to this same end is to conduct a CD melt on this same sample. If only heterodimerization has occurred, then a single transition corresponding to the Tm of the heterodimer will be

observed (this Tm value will probably be different from the value for either of the mixture components). If only homodimerization takes place then two transitions (two Tm's) will be observed.

When peptides of the invention are provided in the form of a multimer, the multimer may be stabilized by covalently joining the peptides, or monomers, to one another. For example, a cysteine residue may be added to either (or both) ends of the monomer and monomers of the multimer covalently joined to one another by a disulfide bond between cysteine residues. Reactions are carried out in accordance with known techniques. In this manner two monomers of a dimer may be covalently joined to form a covalently stabilized dimer, and if desired two such covalently stabilized dimers conjugated to one-another to form a tetramer. In another example, all four members of a tetramer could be covalently joined to one another through disulfide linkages between terminally positioned cysteine residues. Other techniques for stabilizing the multimeric forms of these peptides include crosslinking the monomer components to one another through the formation of intermolecular amide bonds. This process involves the reaction of the amine moiety of a basic amino acid residue (e.g. lysine) with the carboxy moiety of an acidic amino acid residue (e.g. aspartic or glutamic acid).

Preferably, peptides that are analogs of the peptide of SEQ ID NO:1 are antigenic equivalents of the peptide of SEQ ID NO:1. The term "antigenic equivalents" as used herein, refers to proteins or peptides which bind to an antibody which binds to the protein or peptide with which equivalency is sought to be established. Antibodies which are used to select such antigenic equivalents are referred to as "selection antibodies" herein. Examples of such selection antibodies are monoclonal antibodies 4A5 and 6H8 (*see* Hwang *et al.*, Virology 193:924-931 (1993)).

Antigenic equivalents may be formed by modifying reactive groups within a natural sequence or modifying the N-terminal amino and/or C-terminal carboxyl group. Such equivalents include salts formed with acids and/or bases, particularly physiologically acceptable inorganic and organic acids and bases. Other equivalents include modified carboxyl and/or amino groups on the antigen to produce esters or amides, or amino acid protecting groups such a N-t-butoxycarbonyl. Preferred modifications are those which provide a more stable, active peptide which will be less prone to enzymatic degradation in vivo. In general, peptide analogs are 10, 15, 20 or 25 amino acids in length, and may be up to 40, 45, 50, 55 or more amino acids in length.

The selection monoclonal antibodies listed above are specific for conformational epitope(s) displayed by peptides, hence they recognize particularly important epitopes that are useful as components of an antigen to be employed in a diagnostic test for anti-HD.

2. Diagnostic Methods

The diagnostic methods of the present invention provide a method of detecting the presence of antibodies that bind to hepatitis delta antigen in a subject, the presence of which is indicative of a hepatitis delta virus infection in that subject. The method may be performed on mammalian subjects, including human subjects.

Any conventional procedure for detecting antibodies can be employed in practicing the diagnostic assay of the present invention, including agglutination and precipitation reactions, radioimmunoassays, enzyme immunoassays (e.g., U.S. Pat. No. 3,654,090) such as Enzyme-Linked Immunosorbent Assays (ELISA), heterogeneous fluorescent immunoassays (e.g., U.S. Pat. Nos. 4,201,763; 4,171,311; and 3,992,631), and homogeneous (separation-free) immunoassays. See generally *Basic and Clinical*

Immunology, 364-73 (J. Fudenberg et al., eds. 3d Ed. 1980), ELISA is preferred.

In a preferred embodiment, serum or plasma from a subject to be diagnosed is contacted with an antigen (preferably a multimer) as described above so that antibodies in the serum or plasma react in solution with the antigen. While the antigen is preferably bound to a solid support, if a homogeneous (separation-free) immunoassay is utilized to detect the antibodies, a solid support would not be required.

Serum or plasma may be obtained from a human subject generally by pricking a finger and obtaining whole blood (of which serum and plasma are constituents). However, the blood may be processed to obtain only the serum or plasma fraction of the whole blood before contacting the serum or plasma with the bound antigens. Any method for obtaining serum or plasma from a patient may be utilized as long as the antibodies contained therein retain their ability to bind the antigen.

The antigens may be bound to solid supports by known techniques. For example, antigen may be bound by simple electrostatic interactions or a bi-functional organic molecule may be used to attach the antigen to a solid support. The solid support can be made of materials such as plastic (e.g., the bottom surface of a well in a microtiter plate), fiberglass, cellulose acetate and nitrocellulose (e.g., discs). After being attached or adhered to the solid support, the antigens can be cross-linked if desired.

The step of contacting the solid support with a detectable antibody is carried out so that the detectable antibody interacts with the antigen bound to the solid support. The detectable antibody is one which is capable of binding to a human antibody from the serum of the patient which has bound to the purified antigen, where the detectable antibody is capable of being detected. More particularly, the detectable antibody can

be an anti-human immunoglobulin conjugated to a group such as an enzyme which is detectable in the presence of a substrate. Enzyme-conjugated goat, guinea pig, or rabbit anti-human antibodies which have been affinity purified are preferred. In general, the detectable group which is conjugated to the detectable antibody may be any enzyme or other detectable moiety which has been developed for immunoassays. For example, enzymes, fluorescent groups, radioactive groups and others could be used. The enzyme peroxidase is particularly preferred. When peroxidase is the detectable group, a substrate such as 3,3', 5,5'-tetramethylbenzidine or o-phenylenediamine may be used as the substrate for detection of the detectable antibody.

The step of detecting the detectable antibody that has reacted with the human antibodies involves treating or manipulating the detectable group which is conjugated to the detectable antibody to determine its presence. For example, if an enzyme such as peroxidase is conjugated to the antibody, the detecting step would involve adding a peroxidase substrate to the bound antibody, and observing a color change as peroxidase catalyzes conversion of the substrate to a colored species. In the case of other enzymes, such as alkaline phosphatase and β-D-galactosidase, other substrates may be used. The substrate to be used should be chosen such that after the enzyme catalyzes a chemical conversion of the substrate to a product, a change observable to a person employing this test results. Substrates such as 3,3', 5,5'-tetramethylbenzidine, p-nitrophenyl phosphate or 3,3'-diaminobenzidine may be used as substrates. Other detectable groups may also be conjugated to the antibody.

A kit containing the required components for carrying out a diagnostic test based on detection of serum antibodies can be assembled. The kit comprises a package containing purified antigen coated in or on a

solid support such as the bottom of a microtiter plate well or a nitrocellulose or cellulose acetate disc, and a container of a detectable antibody conjugate which is capable of binding antibody from the serum of a patient which is bound to the antigen. An ELISA test is most preferred for the kit since it lends itself to a readily detectable positive or negative diagnosis. Thus, the kit should also house a container of a substrate which is reactive with an enzyme which is conjugated to the detectable antibody, the substrate being readily detectable after reaction with the enzyme. The antigen employed in the diagnostic kit is preferably substantially or essentially free of other proteins. Such kits may optionally contain appropriate control serum or plasma samples that react in a known negative or positive manner in the test.

3. Pharmaceutical Formulations

Peptides of the invention are useful (1) as an immunogen to stimulate immunity to the delta hepatitis virus, (2) as an immunogen to stimulate the production of antibodies to the delta hepatitis virus, (3) as a carrier of other epitopes, and, (4) by virtue of the ability to bind native HDAg, as a component of an effective antiviral peptide or peptide analog for use as a therapeutic agent.

The peptide may be administered to a subject by any suitable means. Examples include intramuscular injection, subcutaneous injection, intravenous infusion, intraperitoneal injection, oral administration, and nasal spray.

The amount of antigen administered will depend upon factors such as the desired effect (i.e., much greater amounts of peptide would be required for use as an antiviral agent than as an immunogen), route of administration, species of subject, and the use and frequency of booster administrations. In general, a

dosage of about 0.1 to about 100 μg per kilogram subject body weight may be used, more particularly about 1 μg per kilogram.

Pharmaceutically acceptable carriers are preferably liquid, particularly aqueous, carriers, such as sodium phosphate buffered saline. The peptide formulation may be stored in a sterile glass container sealed with a rubber stopper through which liquids may be injected and formulations withdrawn by syringe.

As used for immunization purposes (whether to confer protective immunity in a subject or to raise antibodies for use in diagnostic methods), formulations of the present invention may optionally contain one or more adjuvants. Any suitable adjuvant can be used, examples including aluminum hydroxide, aluminum phosphate, plant and animal oils, and the like, with the amount of adjuvant depending on the nature of the particular adjuvant employed. In addition, vaccine formulations may also contain one or more stabilizers, examples including carbohydrates such as sorbitol, mannitol, starch, sucrose, dextrin, and glucose, proteins such as albumin or casein, and buffers such as alkaline metal phosphate and the like.

In the pharmaceutical formulation of the invention the antigen or active agent may be contained within a lipid particle or vesicle, such as a liposome or microcrystal, which may be suitable for parenteral administration. The particles may be of any suitable structure, such as unilamellar or multilamellar, so long as the peptide antigen is contained therein. Positively charged lipids such as N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methyl sulfate, or "DOTAP," are particularly preferred for such particles and vesicles. The preparation of such lipid particles is well known. *See, e.g.*, U.S. Patents Nos. 4,880,635 to Janoff et al.; 4,906,477 to Kurono et al.; 4,911,928 to Wallach;

4,917,951 to Wallach; 4,920,016 to Allen et al.;4,921,757 to Wheatley et al.; etc.

The following examples are provided to more fully illustrate the present invention and should not be construed as restrictive thereof. In these examples, the following abbreviations are used: CD, circular dichroic; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; h, hour; HDAg, hepatitis delta antigen; HDV, hepatitis delta virus; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

EXAMPLE 1

Peptide Synthesis

Three peptides were assembled using solid-phase chemistry and purified by reversed-phase HPLC: δ12-60(Y) (SEQ ID NO:1); δ12-49 (SEQ ID NO:2); and δ25-60(Y) (SEQ ID NO:3). The sequences of these peptides are based in part on the full-length sequence of HDAg from a strain of hepatitis delta virus as reported in the literature (*see* Makino *et al.*, Nature **329**:343 (1987)). Each peptide was N^{α}-acetylated and C^{α}-amidated. Crude peptide in 0.05% trifluoracetic acid/water was separated on an octyl-silica column (C8, Applied Biosystems, 250 mm x 10 mm I.D., 300-Å pore size) by elution at 3 mL/min over 50 min with a linear gradient of 20-42% acetonitrile/water (both containing 0.05% trifluoracetic acid). Peptide δ12-49 was eluted at 32% acetonitrile, δ25-60(Y) at 34.5% acetonitrile, and δ12-60(Y) at 36% acetonitrile (monitored at 230 nm). The homogeneity of the individual fractions was determined using an analytical octyl-silica column.

Peptide δ12-49 (SEQ ID NO:2), consisting of segments A+B of HDAg, included the residues predicted by the computer algorithm of Lupas et al. (*Science*, **252**, 1162-1164 (1991)) to form a coiled-coil, but lacked the 11 residues of segment C. Peptide δ25-60(Y) (SEQ ID NO:3) contained segments B+C but lacked the 13 residues

of segment A. Peptide δ12-60(Y) (SEQ ID NO:1) included segments A, B, and C. Segment B is common to all three peptides, contains three heptads in which the first and fourth heptad positions are occupied by five leucines and one isoleucine, and is probably part of an α-helical coiled-coil. A tyrosine residue, (Y), was present at the C terminus of peptides δ25-60(Y) and δ12-60(Y) to permit radioiodination. This residue is unlikely to contribute to the functional activities of these peptides.

EXAMPLE 2

Circular Dichroic Spectroscopy

CD spectra were recorded with an AVIV Model 60DS CD spectrophotometer using quartz cuvettes (10-mm path). Part (0.5-5 mL) of a stock solution of peptide in water was diluted to 200 mL with phosphate-buffered saline (PBS; 140 mM KCl, 10 mM NaCl, 20 mM sodium phosphate, pH 7.1). Peptide concentrations were determined by quantitative amino acid analysis. The α helicity was estimated (see Chen, Y.-H., et al. (1974) Biochemistry 13: 3350-3359) as $[\theta]222/[\theta]max$, where $[\theta]max$ is the maximal theoretical mean residue ellipticity at 222 nm calculated as $-39,500\ [1-(2.57/n)]$ deg cm^2 $dmol^{-1}$, where n is the number of residues per chain. Thermal denaturation was monitored at 222 nm. Each solution was cooled to 5°C, allowed to equilibrate for 5 min, and spectrally scanned twice using a 15-s averaging time. This procedure was repeated at 5°C intervals until the signal stopped changing, indicating that the structure was fully denatured.

The α helicity and the temperature at the midpoint of thermal denaturation (Tm) of the peptides were determined by CD spectroscopy. All three peptides had high α helicity in PBS at 5°C. The ratio $[\theta 222]/[\theta 208]$ is an indicator of coiled-coil formation. Values close to 1.0 indicate an α-helical coiled-coil and values near 0.8 indicate isolated α helices. *See* Lau, S.

Y. M., *et al.* (1984) *J. Biol. Chem.* 259: 13253-13261. At 5°C this ratio was 0.98 for δ12-60(Y), 0.93 for δ12-49 and 0.88 for δ25-60(Y). At 37°C, this ratio was 0.94 for δ12-60(Y), consistent with persistence of a coiled-coil structure. In contrast, at 37°C this ratio was only 0.79 for δ12-49 and 0.76 for δ25-60(Y), inconsistent with a coiled-coil structure. All three peptides showed protein-like thermal denaturation profiles. At 37°C, peptide δ12-60(Y) was 84% α helical but the shorter peptides were <50% α helical. For peptide δ12-60(Y), the Tm value was much higher (>80°C) and remained higher at a much lower concentration (4 mM) than for the shorter peptides, indicating that both segments A and C are involved in stabilizing its α helical structure. Since Tm increased with peptide concentration each peptide is stabilized by self-association.

EXAMPLE 3

ESI Mass Spectrometry

ESI mass spectra were collected using a Sciex Model API-III mass spectrometer (Thornhill, Ontario) in the positive-ion mode. The sample was infused into the mass spectrometer using a Harvard Model 22 syringe pump. The ion-spray needle was kept at 5,300 V and the orifice potential was held at 80 V. In the deuterium-exchange experiments (*see* Wagner, D. S., *et al.* (1994) *Protein Sci.* 3:1305-1314) each sample was lyophilized to or near dryness to minimize the presence of H_2O. A solution of 5 mM peptide in D_2O (pD 6.5) was infused at 2μL/minute into the enclosed ionization chamber, which was constantly flushed with nitrogen at 4 L/min. Under these conditions, exchange of hydrogen by deuterium occurred only in the solution phase and back exchange of deuterium by hydrogen was negligible in the gas phase. Data collection was begun within 12 s of dissolution. The mass range of m/z 640-1150 containing selected charge states of the molecular ion was scanned repetitively

using a step size of 0.2 Da, a dwell time of 1.5 ms, and a total scan time of 3.4 s. The combined data from several charge states were used to calculate the average molecular mass. The number of hydrogens (*Ht*) remaining to be exchanged at time *t* was calculated as the mass of the fully deuterated molecule minus the observed mass at *t*. Calculation of the number of exchangeable hydrogens and their first-order rate constant for a set of hydrogens undergoing deuterium exchange at the same rate has been described (*see* Wagner, D.S., *et al.*, above).

The stability of the α-helical multimer formed by peptide δ12-60(Y) was studied by determining the kinetics of deuterium exchange. Specifically, the rates of replacement of exchangeable hydrogen atoms by deuterium was measured by ESI mass spectrometry. The lyophilized peptide was dissolved in D_2O (pD 6.5) and the increase in mass due to deuterium exchange monitored as a function of time. Deuterium exchange of peptides δ12-49 and δ25-60(Y) was complete during the 12 s preceding measurement of the first data point, indicating that these peptides are only transiently α-helical at 25°C.

Of the 104 exchangeable hydrogens in the uncharged state of peptide δ12-60(Y), about 41 remained after 12 s, 29 remained after 9 min, and 22 remained after 30 min. These results are consistent with the presence of four kinetically distinct sets of hydrogens: a set of 63 that exchanged very rapidly during the 12 s before the first data point was obtained, a set of 7 that exchanged slowly (first-order rate constant $k = 5.7 \times 10^{-4}$ s^{-1}) over the next 9 min, a set of 12 that exchanged 2.4 times more slowly, and a set of 22 that had not yet exchanged after 30 min, for a total of 41 slowly exchanging hydrogens. If peptide δ12-60(Y) were fully α helical, it would contain 44 NH peptide hydrogens participating in α-helical hydrogen bonds (48 peptide hydrogens (none for Pro^{49}) minus 4 N-terminal NH peptide hydrogens that cannot form α-helical hydrogen bonds).

Therefore, the α helicity of peptide δ12-60(Y) was estimated to be 41/44 or 93% at 25°C as measured by ESI mass spectrometry. For comparison, the α helicity of peptide δ12-60(Y) was estimated to be 97% at 5°C and 84% at 37°C as measured by CD spectroscopy. Thus these two completely different biophysical methods provided very similar values for the α helicity of the δ12-60(Y) multimer. Good agreement of α-helicity estimates by these two methods has been observed previously (*see* Wagner, D. S., *et al.*, cited above).

EXAMPLE 4

Size-exclusion chromatography

The apparent mass of the δ12-60(Y) multimer was determined by comparison to the masses of four globular protein standards: bovine serum albumin, chicken ovalbumin, horse myoglobin, and cytochrome c variant C102T. A TSK-6000 size-exclusion column was eluted with PBS at 0.7 mL/min.

Size-exclusion chromatography confirmed that non-crosslinked δ12-60(Y) formed a multimer with an apparent mass of 30 kDa, which would correspond to a multimer of about five 6-kDa chains. The mass standards were globular proteins but the δ12-60(Y) multimer is likely to have an elongated shape. Thus the apparent mass of the multimer is probably higher than its actual mass, suggesting that the multimer may actually be a tetramer.

EXAMPLE 5

Rate-Zonal Ultracentrifugation and Glutaraldehyde Crosslinking

Further evidence for the self-association of δ12-60(Y) was obtained by rate-zonal ultracentrifugation. Peptides were layered onto 6-20% linear sucrose gradients and centrifuged for 20 h at 36,000 rpm in an SW-41 rotor. Fractions were collected from the bottom of the gradient

and tested for immunoreactivity by ELISA. Peptide δ12-60(Y) sedimented to the center of the 6-20% linear sucrose gradient on centrifugation for 20 h but peptide δ25-60(Y) remained at the top of a similar gradient. Thus δ12-60(Y) formed a large multimer but the shorter peptide δ25-60(Y) did not.

In separate experiments, these two peptides were radiolabeled at the C-terminal tyrosine residue and then chemically crosslinked in the presence of a large quantity of heterogenous serum proteins by brief exposure to low concentrations of glutaraldehyde. The C-terminal tyrosine residues of δ12-60(Y) and δ25-60(Y) were labeled with ^{125}I by the chloramine-T method. A solution of the radiolabeled peptide in PBS containing 2% fetal bovine serum was crosslinked for 3 min with 0.025%, 0.05%, 0.1%, or 0.2% glutaraldehyde. The sample was diluted with Laemmli's buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), and autoradiographed. During separation by sodium dodecyl sulfate-PAGE, crosslinked δ12-60(Y) migrated as a ladder of discrete multimers of increasing mass. The largest crosslinked multimer migrated with an apparent mass of 25 kDa, consistent with the presence of 4 or 5 δ12-60(Y) chains. Although both peptides were randomly crosslinked to larger serum proteins, a similar protein ladder was not obtained with crosslinked δ25-60(Y).

EXAMPLE 6

Peptide Inhibition of HDAg Assembly

The ability of peptide δ12-60(Y) to form multimers suggested that it might form a complex with natural HDAg expressed during HDV infection. To test this possibility, HDAg was extracted under denaturing conditions from the liver of an HDV-infected woodchuck and slowly renatured in the presence of δ12-60(Y) or δ25-60(Y). HDAg from the liver of an acutely infected woodchuck was extracted into 4 M guanidine-HCl, mixed

with 10 mM peptide δ12-60(Y) or δ25-60(Y), and dialyzed against PBS (*see* Wang, J.-G. & Lemon, S. M. (1993) *J. Virol.* **67**: 446-454). The dialysate was subjected to rate-zonal centrifugation in a 10-30% linear sucrose gradient for 18 h at 35,000 rpm in an SW-41 rotor. Fractions were collected from the bottom of the gradient and HDAg immunoreactivity was detected by RIA.

Under these conditions, HDAg normally forms multimers that sediment at 15 S (*see* Wang and Lemon, above). This remained the case when HDAg was renatured in the presence of δ25-60(Y). When renatured in the presence of δ12-60(Y), however, HDAg failed to form 15 S multimers and sedimented below 7 S. Thus δ12-60(Y) inhibited the formation of HDAg multimers. Since HDAg multimers are important for replication of HDV, these results indicate that δ12-60(Y) or related peptides will have significant antiviral activity if delivered to an HDV-infected cell.

EXAMPLE 7

Solid-Phase Immunoassays

An ELISA measured the binding of antibodies to peptide adsorbed directly to a plastic surface. Solutions of peptide (0.64 nM to 2 μM) in 50 mM sodium carbonate buffer (pH 9.0) were incubated overnight at 4°C in quadruplicate wells of a polystyrene microtiter plate. The plate was extensively washed with PBS containing 0.05% Tween-20 detergent and blocked with 10% fetal calf serum in PBS for 25 min at 37°C. Bound peptide was detected by incubation with a human anti-HDAg serum diluted 1:500 in PBS and then with horseradish peroxidase-conjugated guinea pig anti-human IgG antibodies and *o*-phenylenediamine dihydrochloride as substrate. Absorbance was measured at 490 nm.

A sandwich RIA measured the binding of radiolabeled polyclonal anti-HDAg antibodies to peptides captured by anti-HDAg antibodies adsorbed to a plastic

surface. Duplicate wells of a poly(vinyl chloride) microtiter plate were coated with human anti-HDAg serum (1:1000 dilution). Peptide (0.64 nM to 2 μM) in PBS containing 5% fetal calf serum was added. The plate was incubated for 2 h at 37°C and washed with PBS containing 0.05% Tween-20. Bound peptide was detected by adding ^{125}I-labeled human polyclonal anti-HDAg IgG serum (5 x 10^5 cpm/well), washing and counting (*see* Wang, J.-G., *et al.* (1990) *J. Virol.* **64**: 1108-1116).

Epitope mapping studies using short oligopeptides (6-18 residues) from the 12-60 region of HDAg have demonstrated only weak and inconsistent (*i.e.*, not broadly reactive) antigenicity of these peptides. *See* Bergmann *et al.*, *J. Immunol.*, 143: 3714-3721 (1989) and Wang *et al.*, *J. Virol.* **64**:1108-1116 (1990). All three HDAg peptides from the 12-60 region (δ12-49, δ25-60(Y), and δ12-60(Y)) displayed substantial immunoreactivity in an ELISA when probed with a high titer human anti-HD positive serum. Human polyclonal antibodies detected peptides adsorbed to polystyrene from a solution as dilute as 2 μM for δ12-49 and δ25-60(Y) but 0.08 μM for δ12-60(Y). This 25-fold difference in immunoreactivity was not due to different affinities of the peptides for polystyrene because equal amounts of radioiodinated δ25-60(Y) and δ12-60(Y) were bound to the polystyrene. These results indicate that segments A and C both contribute to the immunoreactivity of δ12-60(Y) with antibodies present in this particular serum specimen.

In contrast to these ELISA results, only peptide δ12-60(Y) showed immunoreactivity in a sandwich RIA, in which peptide bound by human anti-HDAg polyclonal antibodies adsorbed to poly(vinyl chloride) was subsequently detected by binding of a radioiodinated polyclonal antibody. A peptide must have at least two epitopes to function as a bivalent ligand in this sandwich RIA, while a peptide that displays only one

epitope can be immunoreactive in the ELISA. Peptide δ12-60(Y) was detected in the sandwich RIA at a concentration as low as 3.2 nM but peptides δ12-49 and δ25-60(Y) were not detected even at 2 μM. Thus only δ12-60(Y) exhibited bivalent binding to polyclonal anti-HDAg antibodies from an HDV-infected patient. This immunoreactivity of δ12-60(Y) correlates with formation of multimers.

EXAMPLE 8

Antigenic Activity of δ12-60(Y)

Plasma samples were obtained from hemophilic patients enrolled in a prospective study of HDV infection. These specimens were tested for the presence of hepatitis B virus envelope protein antigen (HBsAg) and antibodies to HDAg (anti-HD) by commercially available solid-phase immunosorbent assays (ELISAs; Ausria-EIA and Delta-EIA, Abbott Laboratories, N. Chicago, IL). The source of HDag used in the Delta-EIA test was protein extracted from the liver of HDV-infected woodchucks.

Plasma specimens from 89 hemophilic patients were tested for the frequency with which anti-δ12-60(Y) antibodies are present in HDV-infected individuals. Of 23 plasma samples from HBsAg-positive patients with anti-HD antibodies detectable in the commercial anti-HD ELISA utilizing HDAg extracted from woodchuck liver tissue, 22 were strongly positive (absorbance > 2.3) and one was weakly positive (absorbance = 0.53) for anti-δ12-60(Y) activity by peptide ELISA (**FIG. 1A**). In contrast, among plasma samples from 31 HBsAg-negative patients who tested anti-HD negative in the commercial assay, the maximum absorbance was 0.26 (**FIG. 1B**). Of 35 plasma specimens from patients who were HBsAg-positive but anti-HD negative by the commercial ELISA, all but two generated absorbance values less than 0.3 in the peptide ELISA (**FIG. 1C**). The two HBsAg-positive patients with plasma samples demonstrating higher reactivity in the peptide ELISA (absorbance = 0.42 and 0.92) are likely to have had

low levels of anti-HD antibodies not detectable by commercial anti-HD ELISA.

These results demonstrate that peptide δ12-60(Y) displays broadly reactive antigenicity and is specifically recognized by antibodies present in most if not all patients with HDV infection. These data indicate that this peptide has practical use in diagnostic tests for anti-HD antibodies. Because the region spanning residues 12-60 does not contain broadly reactive linear B-cell epitopes that can be successfully modeled with significantly smaller peptides, it is likely that the reactive sites present on peptide δ12-60(Y) include assembled epitopes, which are dependent on this peptide assuming a conformation resembling that of the native molecule.

EXAMPLE 9

Amino Acid Substitutions in the Antigenic Peptides

Alignment of thirteen HDAg peptides is shown in **TABLE 1**. Two peptides have been synthesized: δ12-60(Y) (SEQ ID NO:1) (Makino et al., *Nature* **329**:343 (1987)) and δ12-60(Y)/S22C (SEQ ID NO:4) (not previously described for any natural strain of HDV and containing an engineered substitution (Ser 22 Cys)). Ten additional peptide sequences are derived from HDAg sequences reported in the literature which were obtained from additional strains of hepatitis delta virus (Wang et al., *Nature* **323**:508 and **328**:456 (1986); Imazeki et al., *J. Virol.* **64**:5594 (1990); Chao et al., *Virology* **178**:384 (1990); Kos et al., *J. Med. Virol.* **34**:268 (1991); Saldanha et al., *J. Gen. Virol.* **71**:1603 (1990); Lee et al. *Virology* **188**:265 (1992); Chao et al., *Hepatology* **13**:345 (1991); Casey et al., *Proc. Natl. Acad. Sci. USA* **90**:9016 (1993); Tang et al., *J. Gen. Virol.* **74**:1827 (1993).

These peptides are likely to have related structures and antigenicities despite differences in primary sequence. The alignment shown in **TABLE 1**

indicates that several residues which are present in δ12-60(Y) may be substituted by other residues in other strains of hepatitis delta virus. A consensus sequence is provided as SEQ ID NO:15, and may be the preferred sequence of the peptide.

As shown in **TABLE 1**, Ser-22 of δ12-60(Y) (SEQ ID NO:1) may be substituted by several different amino acids, including alanine, aspartic acid, threonine, asparagine, glycine, and glutamine. This suggests that the composition of this particular residue may not be critical to the structure and antigenicity of the peptide. Other residues are highly conserved (e.g., Pro-Trp-Leu-Gly-Asn).

EXAMPLE 10

Peptide δ12-60(Y) Displays a Conformational Epitope Recognized by Monoclonal anti-HD Antibodies

Four additional peptides were synthesized using methods described in Example 1. δ25-49 (SEQ ID NO:16) represents segment B of the oligomerization domain of HDAg. δ18-49 (SEQ ID NO:17) and δ15-49 (SEQ ID NO:18) represent segment B with an additional 1.0 and 1.5 heptads of segment A, respectively. δ(C)28-60(Y) (SEQ ID NO:19) represents most of segment A plus segment B; a Cys residue has been substituted for Leu^{27} with the hypothesis that under oxidizing conditions disulfide bonds forming between Cys residues of two δ(C)28-60(Y) molecules might be capable of substituting functionally for Leu^{27} and further upstream residues of segments A and B in stabilizing the coiled-coil structure.

TABLE 1

Peptide	Amino Acid Sequence[1]
δ12-60(Y) (SEQ ID NO:1)	GREDILEQW VSGRKKLEEL ERDLRKLKKK IKKLEEDNPW LGNIKGIIGK (Y)
δ12-60(Y)/S22C (SEQ ID NO:4)	GREDILEQW V**C**GRKKLEEL ERDLRKLKKK IKKLEEDNPW LGNIKGIIGK (Y)
δ12-60(Y)/CAR (SEQ ID NO:5)	GRE**E**ILEQW V**A**GRKKLEEL ERDLRK**T**KKK **L**KK**I**ED**E**NPW LGNIKGI**L**GK (Y)
δ12-60(Y)/Fr (SEQ ID NO:6)	GRE**EV**LEQW VSGRKKLEEL ERDLRK**V**KKK IKKLED**EH**PW LGNIKGI**L**GK (Y)
δ12-60(Y)/It1 (SEQ ID NO:7)	GRE**E**ILEQW V**A**GRKKLEEL ERDLRK**T**KKK **L**KK**I**ED**E**NPW LGNIKGI**L**GK (Y)
δ12-60(Y)/It2 (SEQ ID NO:8)	GRE**E**ILEQW V**A**GRKKLEEL ERDLRKLKKK **L**KK**I**ED**E**NPW LGNIKGI**L**GK (Y)
δ12-60(Y)/Ja1 (SEQ ID NO:9)	GRE**Q**ILEQW V**D**GRKKLEEL ERDLRK**I**KKK IKKLEE**E**NPW LGN**V**KGI**L**GK (Y)
δ12-60(Y)/Ja2 (SEQ ID NO:10)	**T**RED**T**LE**K**W **ITA**RKK**A**EEL E**K**DLRKL**R**K**T** IKKLEE**E**NPW LGNI**V**GII**R** (Y)
δ12-60(Y)/Le (SEQ ID NO:11)	GRE**EV**LEQW V**NS**RKK**A**EEL ERDLRK**T**KKK IKKLEEDNPW LGNIKGI**L**GK (Y)
δ12-60(Y)/Na (SEQ ID NO:12)	GRE**EV**LEQW V**G**GR**R**KQEEL ERDLRK**T**KKK IKKLEE**E**NPW LGNIKGI**L**GK (Y)
δ12-60(Y)/Pe (SEQ ID NO:13)	**E**RE**E**ILEQW V**EE**RK**NRRK**L E**K**DLR**RAN**KK IKKLED**E**NPW LGN**VV**G**LLRR** (Y)
δ12-60(Y)/Ta (SEQ ID NO:14)	GRE**EV**LEQW V**N**GRKKLEEL ERDLRK**V**KKK IKKLED**D**NPW LGNIKGI**L**GK (Y)
δ12-60(Y)-Cons (SEQ ID NO:15)	GRE**E**ILEQW V**A**GRKKLEEL ERDLRK**T**KKK IKKLED**E**NPW LGNIKGI**L**GK (Y)

1 - Underlined residues indicate amino acid substitutions from SEQ ID NO:1.

A panel of murine monoclonal antibodies which had been raised to baculovirus-expressed HDAg (*see* Hwang et al., *Virology* **193**:924-931 (1993) were used to test the following peptides for immunoreactivity: δ12-60(Y) (SEQ ID NO:1); δ12-49 (SEQ ID NO:2); δ25-60(Y) (SEQ ID NO:3); δ25-49 (SEQ ID NO:16); δ18-49 (SEQ ID NO:17); δ15-49 (SEQ ID NO:18); and δ(C)28-60(Y) (SEQ ID NO:19). As shown in **TABLE 2**, these peptides represent various segments of the HDAg sequence from amino acid 9-65 (SEQ ID NO:20).

Immunoreactivity was assessed in solid-phase ELISA, as described above, with detection of bound murine antibody using a goat anti-mouse IgG conjugate. Four monoclonal antibodies were evaluated: 4A5 and 6H8 react with a TrpE fusion protein containing residues 11-88 of HDAg, while 3G3 and 8B3 react with a fusion protein containing residues 89-163 of HDAg (*see* Hwang et al., *supra*). 3G3 and 8B3 generate strong reactions in immunoblots of natural HDAg, while 4A5 and 6H8 demonstrate only weak immunoblot reactivity (data not shown). None of these monoclonal antibodies were reactive with nested hexapeptides spanning relevant segments of HDAg, as tested by Multipin Peptide Synthesis epitope scanning (Chiron Mimotypes Pty Ltd., Clayton, Victoria, Australia) (*see also* Geysen et al., *J. Immunol. Methods* **102**, 259-274 (1987)). Monoclonal antibody 8B3 recognized a synthetic peptide representing residues 82-102 of HDAg in a peptide ELISA (data not shown).

TABLE 2

	10 20 30 40 50 60
HDAg (SEQ ID NO:20)	. . . DRGGREDILEQWVSGRKKLEELERDLRKLKKKIKKLEEDNPWLGNIKGIIGKKDKDG . .
Segment (Residues)	<----------><----------><--------->
	A (12-24) B (25-49) C (50-60)
δ12-60(Y) (SEQ ID NO:1)	GREDILEQWVSGRKKLEELERDLRKLKKKIKKLEEDNPWLGNIKGIIGKY
δ25-49 (SEQ ID NO:16)	KKLEELERDLRKLKKKIKKLEEDNP
δ18-49 (SEQ ID NO:17)	EQWVSGRKKLEELERDLRKLKKKIKKLEEDNP
δ15-49 (SEQ ID NO:18)	DILEQWVSGRKKLEELERDLRKLKKKIKKLEEDNP
δ12-49 (SEQ ID NO:2)	GREDILEQWVSGRKKLEELERDLRKLKKKIKKLEEDNP
δ25-60(Y) (SEQ ID NO:3)	KKLEELERDLRKLKKKIKKLEEDNPWLGNIKGIIGKY
δ(C)28-60(Y) (SEQ ID NO:19)	CEELERDLRKLKKKIKKLEEDNPWLGNIKGIIGKY

Note: Double underlining of residues indicate additions/substitutions from HDAg sequence reported by Makino *et al.*, Nature **329**:343 (1987).

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By ELISA, both the 4A5 and 6H8 monoclonal antibodies were strongly reactive with peptide δ12-60(Y) (SEQ ID NO:1), but not with any of the other peptides tested (**FIG. 2A**). Because peptides δ12-49 and δ25-60(Y) together span the entire segment represented by δ12-60(Y) and overlap each other by 25 residues, yet fail to react with either of these monoclonal anti-HD antibodies, the epitopes recognized by these antibodies appear to be assembled structures which are dependent upon the stable coiled-coil assumed by δ12-60(Y). The absence of reactivity of 4A5 and 6H8 antibodies with the oxidized form of δ(C)28-60(Y) (SEQ ID NO:19) may be explained by the fact that the immunoassay involved several steps carried out at 37°C. Although CD spectroscopy shows that oxidized δ(C)28-60(Y) forms a stable coiled-coil at 5°C, this is not the case at 37°C (data not shown). Alternatively, it is possible that the conformational epitopes which are bound by 4A5 and 6H8 directly involve residues upstream of Leu^{27} which are not present in δ(C)28-60(Y). Not surprisingly, antibodies 3G3 and 8B3 which react with a fusion protein containing residues 89-163 of HDAg did not react with any of the peptides tested (**FIG. 2B**).

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Erickson, Bruce W.
Wang, Jia Gang
Rozelle, James E.

(ii) TITLE OF INVENTION: Synthetic Multimeric Peptide with Delta Hepatitis Antigenic Activity

(iii) NUMBER OF SEQUENCES: 20

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Arg Glu Asp Ile Leu Glu Gln Trp Val Ser Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Glu Asp Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Ile Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Arg Glu Asp Ile Leu Glu Gln Trp Val Ser Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Glu Asp Asn Pro
35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Lys Leu Glu Glu Leu Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys
1 5 10 15

Ile Lys Lys Leu Glu Glu Asp Asn Pro Trp Leu Gly Asn Ile Lys Gly
20 25 30

Ile Ile Gly Lys Tyr
35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Arg Glu Asp Ile Leu Glu Gln Trp Val Cys Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Glu Asp Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Ile Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Gly Arg Glu Glu Ile Leu Glu Gln Trp Val Ala Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Thr Lys Lys Lys Leu Lys Lys
20 25 30

Ile Glu Asp Glu Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Arg Glu Glu Val Leu Glu Gln Trp Val Ser Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Val Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Asp Glu His Pro Trp Leu Gly Asn Ile Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Arg Glu Glu Ile Leu Glu Gln Trp Val Ala Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Thr Lys Lys Lys Leu Lys Lys
20 25 30

Ile Glu Asp Glu Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Arg Glu Glu Ile Leu Glu Gln Trp Val Ala Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys Leu Lys Lys
20 25 30

Ile Glu Asp Glu Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly Arg Glu Gln Ile Leu Glu Gln Trp Val Asp Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Ile Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Glu Glu Asn Pro Trp Leu Gly Asn Val Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Thr Arg Glu Asp Thr Leu Glu Lys Trp Ile Thr Ala Arg Lys Lys Ala
1 5 10 15

Glu Glu Leu Glu Lys Asp Leu Arg Lys Leu Arg Lys Thr Ile Lys Lys
20 25 30

Leu Glu Glu Glu Asn Pro Trp Leu Gly Asn Ile Val Gly Ile Ile Arg
35 40 45

Tyr

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Arg Glu Glu Val Leu Glu Gln Trp Val Asn Ser Arg Lys Lys Ala
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Thr Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Glu Asp Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Arg Glu Glu Val Leu Glu Gln Trp Val Gly Gly Arg Arg Lys Gln
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Thr Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Glu Glu Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Glu Arg Glu Glu Ile Leu Glu Gln Trp Val Glu Glu Arg Lys Asn Arg
1 5 10 15

Arg Lys Leu Glu Lys Asp Leu Arg Arg Ala Asn Lys Lys Ile Lys Lys
20 25 30

Leu Glu Asp Glu Asn Pro Trp Leu Gly Asn Val Val Gly Leu Leu Arg
35 40 45

Arg Tyr
50

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Arg Glu Glu Val Leu Glu Gln Trp Val Asn Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Val Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Asp Asp Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Gly Arg Glu Glu Ile Leu Glu Gln Trp Val Ala Gly Arg Lys Lys Leu
1 5 10 15

Glu Glu Leu Glu Arg Asp Leu Arg Lys Thr Lys Lys Lys Ile Lys Lys
20 25 30

Leu Glu Asp Glu Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Leu Gly
35 40 45

Lys Tyr
50

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Lys Lys Leu Glu Glu Leu Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys
1 5 10 15

Ile Lys Lys Leu Glu Glu Asp Asn Pro
20 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Glu Gln Trp Val Ser Gly Arg Lys Lys Leu Glu Glu Leu Glu Arg Asp
1 5 10 15

Leu Arg Lys Leu Lys Lys Lys Ile Lys Lys Leu Glu Glu Asp Asn Pro
20 25 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Asp Ile Leu Glu Gln Trp Val Ser Gly Arg Lys Lys Leu Glu Glu Leu
1 5 10 15

Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys Ile Lys Lys Leu Glu Glu
20 25 30

Asp Asn Pro
35

---

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Cys Glu Glu Leu Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys Ile Lys
1 5 10 15

Lys Leu Glu Glu Asp Asn Pro Trp Leu Gly Asn Ile Lys Gly Ile Ile
20 25 30

Gly Lys Tyr
35

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Asp Arg Gly Gly Arg Glu Asp Ile Leu Glu Gln Trp Val Ser Gly Arg
1 5 10 15

Lys Lys Leu Glu Glu Leu Glu Arg Asp Leu Arg Lys Leu Lys Lys Lys
20 25 30

Ile Lys Lys Leu Glu Glu Asp Asn Pro Trp Leu Gly Asn Ile Lys Gly
35 40 45

Ile Ile Gly Lys Lys Asp Lys Asp Gly
50 55

THAT WHICH IS CLAIMED IS:

1. A synthetic peptide having delta hepatitis virus antigenic activity selected from the group consisting of:

(a) the synthetic peptide δ12-60(Y), said peptide having the sequence:

Gly-Arg-Glu-Asp-Ile-Leu-Glu-Gln-Trp-Val-Ser-Gly-Arg-Lys-Lys-Leu-Glu-Glu-Leu-Glu-Arg-Asp-Leu-Arg-Lys-Leu-Lys-Lys-Lys-Ile-Lys-Lys-Leu-Glu-Glu-Asp-Asn-Pro-Trp-Leu-Gly-Asn-Ile-Lys-Gly-Ile-Ile-Gly-Lys-Tyr (SEQ ID NO:1);

(b) a synthetic peptide selected from the group consisting of δ12-60(Y)/S22C (SEQ ID NO:4), δ12-60(Y)/CAR (SEQ ID NO:5), δ12-60(Y)/Fr (SEQ ID NO:6), δ12-60(Y)/It1 (SEQ ID NO:7), δ12-60(Y)/It2 (SEQ ID NO:8), δ12-60(Y)/Ja1 (SEQ ID NO:9), δ12-60(Y)/Ja2 (SEQ ID NO:10), δ12-60(Y)/Le (SEQ ID NO:11), δ12-60(Y)/Na (SEQ ID NO:12), δ12-60(Y)/Pe (SEQ ID NO:13), δ12-60(Y)/Ta (SEQ ID NO:14), and δ12-60(Y)-Cons (SEQ ID NO:15); and

(c) peptides at least 40 amino acids in length that form a heteromer or a homomer with the peptide δ12-60(Y).

2. A protein comprising a homomer or a heteromer of a peptide of claim 1.

3. A protein according to claim 2, wherein said homomer or heteromer is a dimer, tetramer, hexamer, or octamer, or aggregates thereof.

4. A protein according to claim 2, which protein specifically binds to a combining site of an antibody, which combining site specifically binds to a conformationally determined epitope of hepatitis delta virus antigen.

5. A method for detecting, in a mammalian subject, the presence of antibodies that bind to hepatitis delta virus antigen, comprising the steps of:

contacting a biological sample taken from said subject with an antigen comprising a peptide selected from the group consisting of δ12-60(Y) (SEQ ID NO:1), δ12-60(Y)/S22C (SEQ ID NO:4), δ12-60(Y)/CAR (SEQ ID NO:5), δ12-60(Y)/Fr (SEQ ID NO:6), δ12-60(Y)/It1 (SEQ ID NO:7), δ12-60(Y)/It2 (SEQ ID NO:8), δ12-60(Y)/Ja1 (SEQ ID NO:9), δ12-60(Y)/Ja2 (SEQ ID NO:10), δ12-60(Y)/Le (SEQ ID NO:11), δ12-60(Y)/Na (SEQ ID NO:12), δ12-60(Y)/Pe (SEQ ID NO:13), δ12-60(Y)/Ta (SEQ ID NO:14), and δ12-60(Y)-Cons (SEQ ID NO:15), under conditions permitting said antigen to specifically bind an antibody in the sample to form an antibody-antigen complex; and then

determining the amount of antibody-antigen complex in the sample as a measure of the amount of antibody in the sample.

6. A method of screening a mammalian subject for the presence of hepatitis delta virus infection, comprising the method of claim 5, wherein an elevated level of antibody in said sample is associated with the presence of hepatitis delta virus infection.

7. The method of claim 5 wherein said mammalian subject is a human.

8. The method of claim 5 wherein said biological sample is selected from the group consisting of blood, serum and blood plasma.

9. A method according to claim 5, which method is selected from the group consisting of radioimmunoassay, immunofluorescence assay, and enzyme immunoassay.

10. A kit useful for detecting hepatitis delta virus antibodies in a subject, comprising an antigen according to claim 1.

11. A kit according to claim 10, further comprising a solid support, wherein said antigens are immobilized on said solid support.

12. A method of producing antibodies to delta hepatitis virus in a mammalian subject, comprising administering to said subject an immunogenic amount of a peptide of claim 1.

13. The method of claim 12 wherein said antibodies are neutralizing antibodies.

14. A method of immunizing a mammalian subject against delta hepatitis virus infection comprising administering to the subject an immunogenic amount of a peptide of claim 1.

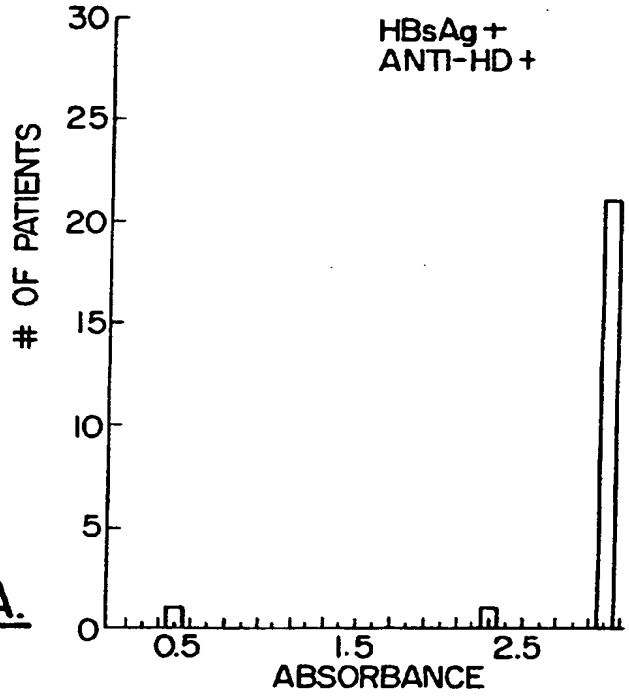
HBsAg+
ANTI-HD+
OF PATIENTS
30
25
20
15
10
5
0
0.5
1.5
2.5
ABSORBANCE

FIG. 1A.

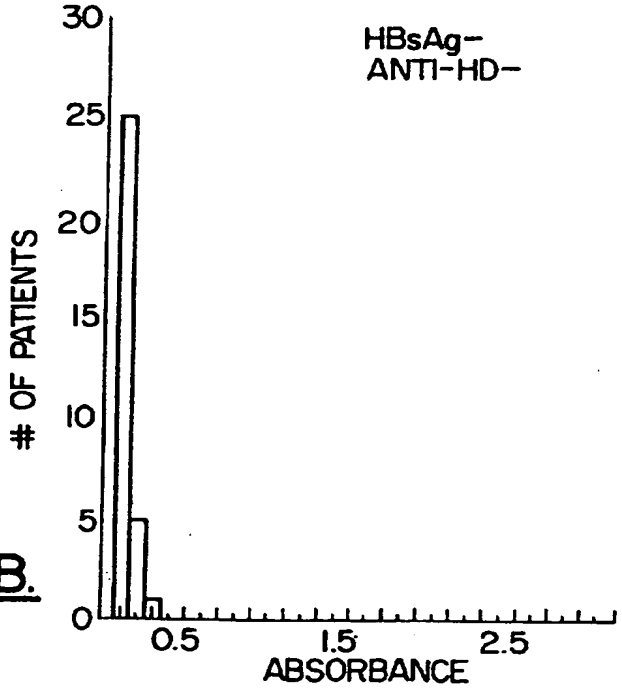
HBsAg−
ANTI-HD−
OF PATIENTS
30
25
20
15
10
5
0
0.5
1.5
2.5
ABSORBANCE

FIG. 1B.

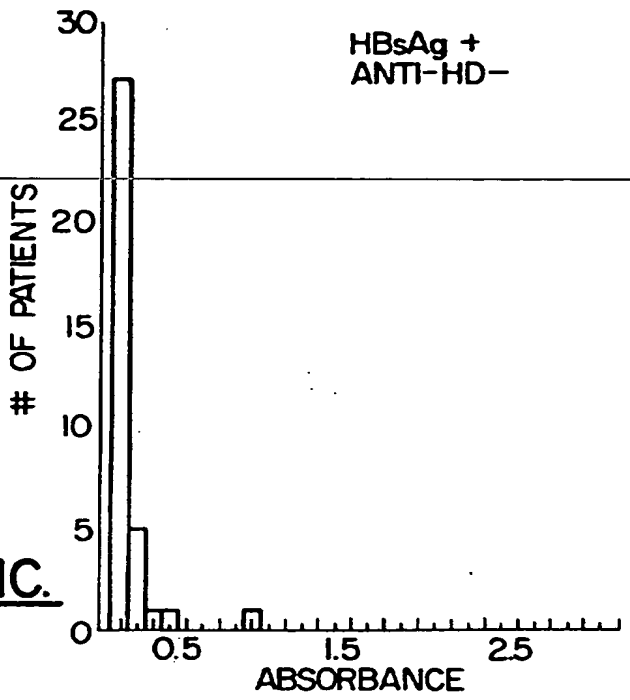

FIG. IC.

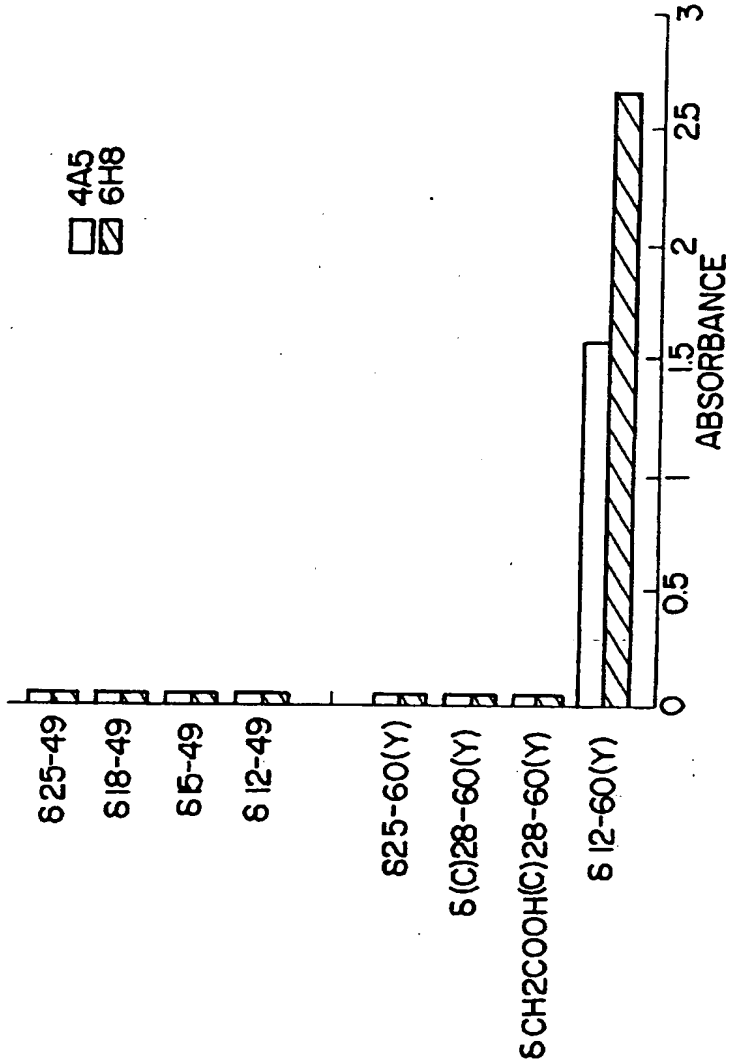
4A5
6H8
ABSORBANCE
0
0.5
1
1.5
2
2.5
3
δ25-49
δ18-49
δ5-49
δ12-49
δ25-60(Y)
δ(C)28-60(Y)
δCH2COOH(C)28-60(Y)
δ12-60(Y)

FIG. 2A.

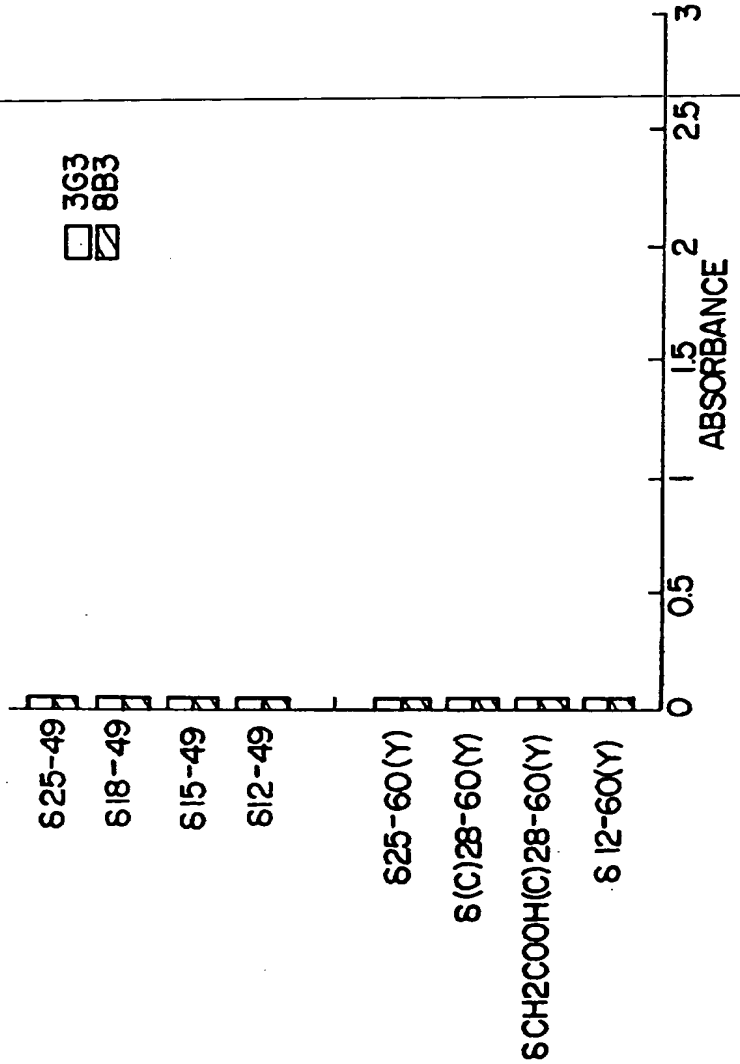
3G3
8B3
ABSORBANCE
0
0.5
1
1.5
2
2.5
3
δ25-49
δ18-49
δ15-49
δ12-49
δ25-60(Y)
δ(C)28-60(Y)
δCH2COOH(C)28-60(Y)
δ12-60(Y)

FIG. 2B.